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## COMBINED CHEMOTHERAPY OF TUBERCULOSIS WITH KANAMYCIN AND CYCLOSERINE

### I. THE ANTITUBERCULOUS ACTIVITY OF COMBINED USE OF KANAMYCIN AND CYCLOSERINE *IN VITRO* AND IN ANIMALS

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#### Introduction

At present one of the most cumbersome problems is to treat serious pulmonary tuberculosis patients for whom surgical operations are not efficacious and whose bacilli have become resistant to Streptomycin (SM), Para-aminosalicylic acid (PAS) and Isonicotinic acid hydrazide (INH).

In 1954, a new antibiotic substance, Cycloserine (CS) was isolated by Harned and associates<sup>1,2,3)</sup> from cultures of *Streptomyces orchidaceus*. This antibiotic shows definite tuberculostatic activity *in vitro* and *in vivo*, though not so powerful as SM or INH.

In 1955, another new antibiotic substance, Kanamycin (KM) was isolated by Umezawa and associates<sup>4,5)</sup> from cultures of *Streptomyces kanamyceticus*, and was clearly demonstrated to have nearly equal tuberculostatic activity to SM.<sup>6)</sup>

The combined use of KM and CS would be expected to bring about favorable effect on such serious pulmonary tuberculosis.

In this paper, the results of the experimental studies on the antituberculous activity of KM-CS *in vitro* and in animals will be reported.

#### Experiments

(I) The tuberculostatic activity of KM-CS *in vitro*.

##### Methods:

The minimal inhibitory concentration (MIC) of KM-CS was compared with that of KM or CS alone.

The culture medium employed was Kirchner's liquid medium containing bovine serum in the concentration of 10 per cent. The pH, when required, was adjusted to 5.5, 6.5 and 7.5.

Serial dilutions of the drugs were made with the Kirchner's media.

Tubes with 2 ml. of the media containing serially diluted drugs and control tubes were inoculated with tubercle bacilli (H37Rv) in the amount of 0.1 mg., 0.01 mg. or 0.001 mg. per ml. of the media.

All tubes were incubated at 37°C. At the end of the first month, the tubes were examined and the MIC was recorded.

#### Results :

Table 1 shows the results of KM-CS used at the rate of each clinical dose (1 to 1). The MIC of KM increased with the increase of the size of inocula and also in acid media while that of CS was less influenced by these factors. The MIC of KM-CS decreased to one half of that used alone when a larger inoculum (0.1 mg.) was used in alkaline media.

**Table 1.** Tuberculostatic Effect of Kanamycin-Cycloserine at the Combination Rate of Each Clinical Dose (1 to 1).

Inoculum Size	Materials	Experiment I						Experiment II					
		pH 5.5		pH 6.5		pH 7.5		pH 5.5		pH 6.5		pH 7.5	
		KM	CS	KM	CS	KM	CS	KM	CS	KM	CS	KM	CS
0.1 mg.	KM	5.0		2.5		1.25		5.0		2.5		1.25	
	CS		12.5		12.5		12.5		12.5		12.5		12.5
	KM-CS	2.5	2.5	2.5	2.5	0.625	0.625	2.5	2.5	0.625	0.625	1.25	12.5
0.01 mg.	KM	1.25		1.25		0.625		1.25		1.25	12.5	0.625	
	CS		12.5		12.5		12.5		12.5				12.5
	KM-CS	1.25	1.25	0.625	0.625	0.313	0.313	1.25	1.25	0.625	0.625	0.625	0.625
0.001 mg.	KM	0.625		0.625		0.313		0.625		0.625		0.625	
	CS		12.5		12.5		12.5		12.5		12.5		12.5
	KM-CS	0.625	0.625	0.625	0.625	0.313	0.313	0.625	0.625	Contaminated		0.625	0.625

Numerals indicate the MIC in  $\gamma$  per ml.

**Table 2.** Tuberculostatic Effect of Kanamycin-Cycloserine at the Combination Rate of Each Minimal Inhibitory Concentration (1 to 10).

Materials	Experiment 1						Experiment 2					
	0.1 mg.		0.01 mg.		0.001 mg.		0.1 mg.		0.01 mg.		0.001 mg.	
	KM	CS	KM	CS	KM	CS	KM	CS	KM	CS	KM	CS
KM	2.5		1.25		1.25		2.5		1.25		1.25	
CS		12.5		12.5		12.5		25.0		25.0		25.0
KM — CS	0.625	6.25	0.625	6.25	0.625	6.25	0.625	6.25	0.313	3.13	0.313	3.13

Numerals indicate the MIC in  $\gamma$  per ml.

Table 2 shows the results of KM-CS used at the rate of each MIC (1 to 10). KM-CS demonstrates the synergistic effect to a certain extent in every inoculum size in this experiment.

- (II) Development of the resistance of *Mycobacterium tuberculosis* to KM and CS transferred successively in media containing fixed doses of the drugs.

Methods :

Tubes with 2 ml. of Kirchner's medium containing serially diluted concentrations of KM, CS and/or KM-CS (at the rate of 1 to 1) were inoculated with 0.1 mg. of tubercle bacilli (H37Rv).

Three tubes were used for each concentration of the drugs.

After one month's incubation at 37°C, the supernatant was removed aseptically and the sediment of one of these three tubes was used for the sensitivity test to KM and CS employing the egg media.

The sediments of the other tubes were resuspended in newly prepared media containing the same concentration of the drugs as the former ones in order to prevent the decrease of potency of the drugs as possible.

Results :

The results are shown in table 3. When the bacilli were incubated in a medium containing KM in the concentration of 100 $\gamma$  per ml., the bacterial resistance

**Table 3.** Development of Resistance of H37Rv to Kanamycin and Cycloserine in Media Containing Fixed Concentrations of Drugs.

Concentration of Drug		Drug Sensitivity after					
		1 Month***		2 Months		3 Months	
KM	CS	KM	CS	KM	CS	KM	CS
100 $\gamma$		10.0*		n.G.**		n.G.	
10 $\gamma$		5.0		50.0		50.0	
1 $\gamma$		1.25		10.0		50.0	
	100 $\gamma$		6.25		12.5		12.5
	10 $\gamma$		12.5		12.5		12.5
	1 $\gamma$		6.25		6.25		12.5
100 $\gamma$	100 $\gamma$	n.G.	n.G.	n.G.	n.G.	n.G.	n.G.
10 $\gamma$	10 $\gamma$	5.0	12.5	5.0	12.5	n.G.	n.G.
1 $\gamma$	1 $\gamma$	2.5	6.25	2.5	6.25	2.5	12.5

\* Numerals indicate the MIC in  $\gamma$  per ml.

\*\* n.G. means no growth of the incubated organisms. Therefore sensitivity test was not performed.

\*\*\* Renewal of the media (drugs) was performed at the end of each month.

to KM developed to 10 $\gamma$  per ml. after one month.

After 2 months the drug-sensitivity test to KM was not determined because the growth of the organisms was not observed even in the control egg media (without KM). This may be due to the bactericidal effect of the drug.

When the bacilli were incubated in media containing KM in lower concentrations, the resistance of the organisms to KM developed rapidly up to 50 $\gamma$  per ml. after two successive transfers.

When the organisms were inoculated in media containing KM or CS each in the concentration of 100 $\gamma$  per ml., no growth was found after one month. This might be due to the enhanced bactericidal activity of KM by the addition of CS.

When the organisms were inoculated into media containing lower concentrations of KM-CS, their growth was observed but no development of drug-resistance was recognized even after two successive transfers.

(III) The tuberculostatic activity in the serum of the rabbits after the administration of KM-CS.

#### Methods :

Five rabbits, each weighing approximately 3 kg. were employed and they were fasted on the day of drug administration.

The test dosage of CS (20 mg. per kg.) was dissolved in 5 ml. of water and poured into the stomach of the rabbits by Nelaton's catheter, and that of KM (20 mg. per kg.) was injected intramuscularly in the back.

The blood samples for the test were collected aseptically before the drug-administration as controls and 1, 3, 5, 7 and 9 hours after the administration of the drugs and allowed to stand for 24 hours.

Then the sera were separated from these samples by centrifugation, and the modified Kirchner's media added with sera in the concentration of 90 per cent were prepared by Shioda's method<sup>7)</sup> for each series of the experiment.

The medium thus obtained is composed of 0.1 ml. of the modified Kirchner's basal medium and 0.9 ml. of the serum.

In the modified Kirchner's basal medium, all ingredients were added to 10 times of the original concentration.

These media (1.0 ml.) were inoculated with 0.025 mg. of H37Rv and were incubated at 37°C for four weeks.

#### Results :

As may be seen in Table 4, the tuberculostatic activity of the sera was recognized up to 5 or 7 hours after administration of KM alone. The combined use of KM and CS resulted in a longer antituberculous effect compared with the single use of KM.

**Table 4.** Duration of Bacteriostatic Activity of Sera after Administration of Drugs.

Rabbit No.	Kanamycin 20 mg. per kg.	Kanamycin 20 mg. per kg. and Cycloserine 20 mg. per kg.
A 1	up to 7 hours	up to 7 hours
A 2	" 5 "	" 7 "
A 3	" 5 "	" 7 "
A 4	" 5 "	" 7 "
A 5	" 7 "	" 9 "

(IV) The antituberculous activity of KM-CS on the experimental tuberculosis.

A) The experiment with ocular tuberculosis of guinea pigs.

Methods :

Guinea pigs, each weighing approximately 500 g. and negative to tuberculin skin reaction (0.1 cc. of 1:100 diluted O.T.), were inoculated subcutaneously in the groin with 0.1 mg. (wet weight) of H37Rv. Four weeks after inoculation,

**Table 5.** Index of Experimental Tuberculous Lesions in the Anterior Segments of the Guinea Pigs Eyes.

Findings		Index of Tuberculous Lesions					
		I	II	III	IV	V	VI
Conjunctiva	Ciliary Injection	÷	+	++	+++	+++	Perforation or Phthisis bulbi
	Edema	—	—	÷	—	++	
Cornea	Curvature	—	—	—	—	+	
	Dullness	—	+	+	++	+++	
	Vascularization	—	+	+	++	+++	
Anterior Chamber	Depth	normal	various	various	various	unclear	
	Turbidity	—	+	+	++	+++	
	Puruloid Substance	—	—	+	++	+++	
Iris	Irregularity	+	++	+++	+++	unclear	
	Hyperemia and Swelling	+	++	+++	+++		
	Vasodilatation	—	+	++	+++		
	Tubercles	—	various	various	various		
Pupil	Form	round	various	various	various	unclear	
	Tubercles	—	various	various	various		
	Light Reflex	normal	various	various	various		

when the positive conversion of tuberculin skin reaction was confirmed in all guinea pigs, the anterior chambers of their right eyes were punctured and inoculated with 0.002 mg. (wet weight) of the same strain in order to produce the experimental ocular tuberculosis. The development of the ocular tuberculous lesions were precisely followed by means of hand slit-lamp examination and recorded the index of lesions of each animal.

The severity of the tuberculous lesions in the anterior segment of the eye was graded by the index of W. Steenken Jr. et al.<sup>8)</sup>, modified by M. Naito et al.<sup>9)</sup> as shown in Table 5.

When the moderately advanced tuberculous ocular lesions were observed, a week after the intraocular inoculation, the animals were divided into three groups. Each group involved six guinea pigs and the arithmetic mean of the indices was regarded as the index of the group at that time.

Group 1: KM 20 mg./kg. three times a week (subcutaneously)

Group 2: KM 20 mg./kg. three times a week (subcutaneously) +  
CS 10 mg./kg. daily (orally)

Group 3: Control

The administration of the drugs started on the day of grouping the animals and continued for eight weeks. The mean values of tuberculosis indices of the ocular lesions in each group were plotted successively.

#### Results :

As shown in Fig. 1, though KM used with CS in the treatment of ocular tuberculosis of guinea pigs seemed to be effective, marked difference in the therapeutic effect between KM-CS and KM alone was not observed.

B) The experiment with the survival time of tuberculous mice administered KM and/or CS.

#### Methods :

Inbred female mice (dd-strain), each weighing 18 to 20 gm., were inoculated intravenously with 0.5 mg. (wet weight) of *Mycobacterium tuberculosis var. hominis* (KURONO strain, isolated by K. Mizunoe<sup>10)</sup>) and divided into five groups as follows :

Group 1: KM 20 mg./kg. twice a week, subcutaneously

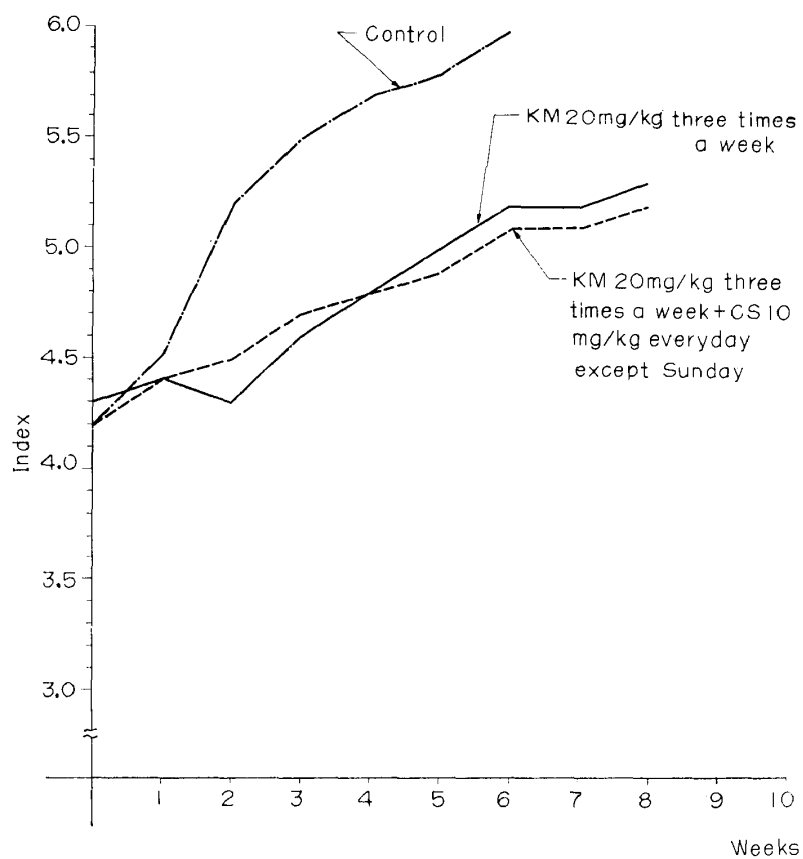
Group 2: CS 10 mg./kg. daily, orally

Group 3: KM 10 mg./kg. twice a week + CS 5 mg./kg. every day

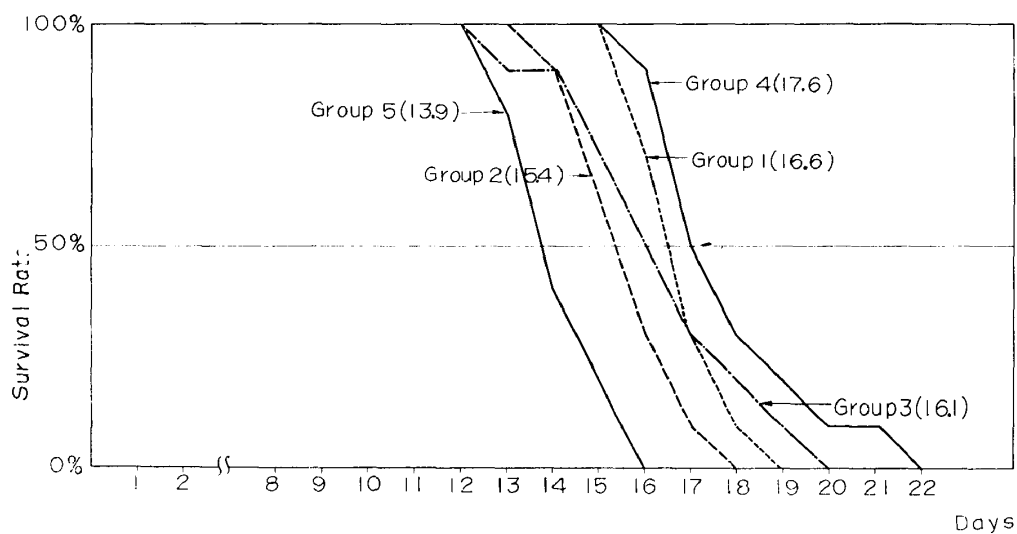
Group 4: KM 20 mg./kg. twice a week + CS 10 mg./kg. every day

Group 5: Control

**Figure 1.** The grade of the Tuberculose Lesions in the Anterior Segment of Guinea Pig's Eyes.



**Figure 2.** Survival Rate of Tuberculous Mice treated with KM and CS.



Numerals in parenthesis indicate mean survival time in days.



The administration of the drugs started on the following day of the intravenous inoculation of tubercle bacilli and was kept along until a half of mice of any one of the treated groups were dead. The number of dead mice was plotted on the graph.

#### Results :

In this experiment, KM 20 mg./kg. used with CS 10 mg./kg. was most effective (Fig. 2). And KM 10 mg./kg. used with CS 5 mg./kg. showed slightly less effective than KM 20 mg./kg. alone. Mean survival time in days is shown in Fig. 2.

### Discussion and Summary

It is recognized that pulmonary tuberculosis patients who are excreting tubercle bacilli resistant to SM, PAS and/or INH, have been increasing in number. Though this may be caused by failure of previous chemotherapy or by infection with drug-resistant tubercle bacilli, the treatment to these patients is one of the most difficult tasks in the tuberculosis clinic.

In the experiments described above, it was shown that KM used with CS exhibited the definite synergistic effects *in vitro* and the development of bacterial resistance to KM was evidently prolonged due to the combined use of KM and CS.

The fact that the MIC of KM is much more influenced by the size of inoculum and the pH of culture media than is CS, should be noted. The apparent similarity of the effects of pH and inoculum size on KM and SM deserves further investigation.

The bacteriostatic activity of the serum after the administration of KM-CS was maintained longer than that of KM alone which may indicate the availability of the combined use of these two drugs in tuberculosis clinics.

Although the survival rate of tuberculous mice administered both KM and CS showed a remarkable synergistic effect, the influence of the combined use of these two drugs on the ocular tuberculous lesions of guinea pigs was not remarkably better than that of KM or CS alone. As for the discrepancy between the results of these two experiments in animals, it might be attributable to the differences in the size of inoculum and the dosage of the drugs administered.

The clinical effect of the regimen of KM and CS in the treatment of serious pulmonary tuberculosis will be reported in the near future.

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